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REGULATION AND FUNCTION OF CYTOKINES THAT PREDICT PROSTATE CANCER METASTASIS

PRINCIPAL INVESTIGATOR:
NEIL A. BHOWMICK

CONTRACTING ORGANIZATION:
Cedars-Sinai Medical Center
Los Angeles, CA 90048

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E-Mail: bhowmickn@cshs.org

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14. ABSTRACT

Patients at high-risk for metastatic progression of prostate cancer are unfortunately treated too late. We identified biomarkers that help distinguish aggressive disease from those that do not progress following prostatectomy. Specifically, CX3CL1 and IL15 were identified to be down regulated in subjects that developed recurrent prostate cancer. The studies presented suggest that both CX3CL1 and IL15 significantly reduce the motility of prostatic epithelia (LNCaP), but interestingly had little effect on non-tumorigenic prostatic cells (BPH1). Further, these two cytokines similarly reduced adhesion of LNCaP cells to collagen I. However, sensitivity to aniokis was dramatically induced in the same cells by CX3CL1 and IL15. This data support the clinical observation of recurrent free subjects having greater expression of CX3CL1 and IL15. Further, these factors may even serve as anti-metastatic mediators, despite their limited effects on tumor cell proliferation.

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Progress Report: Regulation and Function of Cytokines That Predict Prostate Cancer Metastasis

a. INTRODUCTION

There is a large disparity between the number of newly diagnosed cases of prostate cancer in the United States every year and the number of men who die of the disease. The 30,000 deaths annually in the US caused by prostate cancer are almost entirely due to metastatic progression [1]. As a consequence, even though prostate cancer is the second leading cause of cancer related mortality in men in the United States, there is an ongoing concern that as a medical community we are over diagnosing, and hence over treating, the disease. *Yet, patients at high-risk for metastatic progression are unfortunately treated too late*. The challenge has been to determine up-front which patients harbor high-risk disease requiring aggressive/curative therapy and which patients harbor indolent disease that could be managed with active surveillance. The issue is an important one given the potential for attempts at local curative therapy (whether it be surgery, radiation or cryotherapy) to subject the patient to both short-term and long-term morbidity. Currently clinicians rely on a combinatorial assessment of the pre-treatment PSA value, clinical tumor stage, and biopsy-Gleason score to risk stratify patients. These methods are unable to distinguish 80% of the patients that may not have any clinical consequences from the prostate cancer [2]. Next, following local curative therapy the issue of requirement and timing for second line adjuvant therapy becomes increasing important. However, treatment of cancers prior to metastatic progression with conventional chemotherapy has shown promise of late [3-5]. It is critical to accurately determine the appropriate candidates for such adjuvant therapy given the potential for decreased quality of life and added morbidity associated with chemotherapy treatment, especially since the majority (65%) of patients remain disease free after prostatectomy. Since men experiencing PSA recurrence following surgical treatment suggest metastatic spread of the disease, better forms of early detection and risk stratification would support targeted use of adjuvant therapies [6]. Similar to the clinical stratification described for patients prior to primary treatment for prostate cancer, pathologic risk of biochemical recurrence is performed following primary treatment. One of the most commonly used nomogram for post-operative predictions has been described by Kattan and colleagues [7, 8]. Multiple criteria that include the pre-treatment PSA, prostate capsule invasion, pathologic Gleason score, surgical margin status, seminal vesicle involvement, and lymph node involvement for predicting post-operative biochemical recurrence [7, 8]. However, a model of such clinical/pathologic parameters is limited (particularly at the level of sensitivity) by the fact we do not know all the predictive factors.

Chemokines, cytokines, and growth factors in the tumor microenvironment regulate the fate of tumor progression [9, 10]. We hypothesized that tissue chemokines can be strong biomarker candidates for distinguishing patients with high risk for biochemical recurrence or metastatic progression of prostate cancer. CX3CL1 exhibited the best prediction ability ($P < 0.0001$) followed by CCL4 ($P < 0.001$) and IL-15 ($P = 0.003$). The proportional hazard assumption was tested with scaled Schoenfeld residuals [11]. There was no evidence of violation as the chi-square tests for trend were not significant for any of the seven variables (surgical margin status, seminal vesicle involvement, Gleason Score, pre-operative PSA, CCL4, and CX3CL1, and IL-15; P values ranging 0.46 to 0.90). The same two chemokines, CCL4 ($P=0.040$) and CX3CL1 ($P<0.0001$) were significant factors. In addition, pre-operative PSA ($P= 0.0025$) and surgical margin ($P= 0.023$) were significant [12]. We described a strong predictive ability of differentially expressed chemokines in a nested case-control study of prostate cancer patients that develop biochemical recurrence or lead recurrent-free lives following prostatectomy. The goal of this proposal is to determine the biologic role of these potentially clinically relevant chemokines in prostate cancer progression.

In this past year, we focused on the role of stromally derived IL15 and CX3CL1 - the two cytokines that are downregulated in PCa patients with recurrent disease. However, based on our past findings on the recruitment of bone marrow derived cells (BMDCs) and the rapid prostatic proliferation during CRPC-associated regrowth. Tissue remodeling, and cancer progression are generally associated with the recruitment of BMDCs [13]. Co-expression of prostate markers with BMDCs suggested that these recruited cells were also incorporated into the prostate epithelia [14]. We further identified MSCs fusing with prostatic epithelia. Interestingly, the chemokine, CCL4, up regulated in patients with biochemical recurrence, recruits MSC. CX3CL1, down regulated in the recurrent population, is critical to the communication with MSC in eliciting anti-tumor activity. However, the goals this year was to characterize the paracrine (not systemic) role of IL15 and CX3CL1 on prostatic epithelia.

b. **BODY**

The data described here is work done as a result of this DOD award. The focus of **Aim 1** was to determine the effect of the differentially expressed chemokines on prostate cancer cells. Our knowledge of the importance of the tumor microenvironment in prostate cancer metastatic progression is addressed in **Aim 2** of the proposal. In the last progress report we discussed progress on the biologic role of CCL4 in prostate cancer progression through the development of in vitro and in vivo models.

Of the three chemokines: CX3CL1, CCL4, and IL-15, that helped distinguish prostate cancer patients that developed recurrent disease [12], we chose to determine the role of CX3CL1 and IL15 this past year. We used the Amaxa neucleofection system (Lonza) as opposed to the originally proposed lenti viral system to overexpress the genes of interest in the prostatic fibroblastic cells. While lenti systems have good transduction efficiency, we found neucleofection to be superior, with approximately 80% efficiency (**Figure 1**). Following blastasidine selection, the fibroblastic cells expressed significantly greater CX3CL1 and IL15, compared to their parental control, by rtPCR (**Figure 1**). Western blot verification of the protein expression could not be done, as appropriate anitbodies were not available. The conditioned media from prostatic stromal fibroblastic cells that expressed GFP control vector, CX3CL1, or IL15 were transferred to LNCaP or BPH1 cells in 60 mm dishes. Sequential cell counting was performed for 3 days to determine the role of stromally derived CX3CL1 and IL15. The results suggested, a trend of reduced proliferation in both tumorigenic and non-tumorigenic cell types, respectively (**Figure 2**). However, statistical significance was not reached in either cell type.

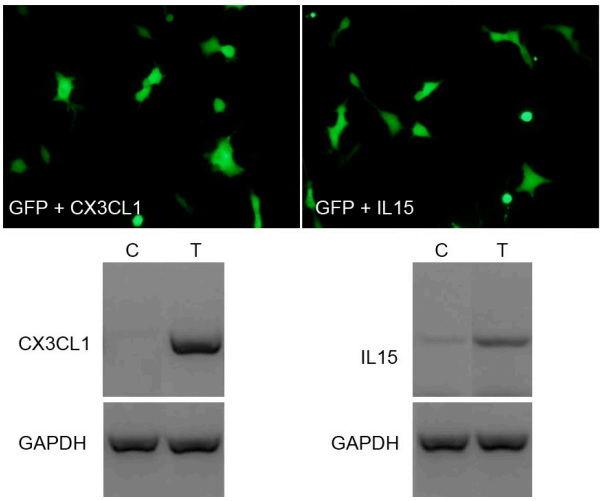

Figure 1. Prostatic fibroblasts were transfected with CX3CL1 and IL15 expression constructs followed by blasticidine selection. A GFP vector was introduced one fifth of either CX3CL1 or IL15 constructs to monitor transfection efficiency (upper panels). RT PCR for CX3CL and IL15 expression in transfected (T) cells were compared to GFP only transfected control (C, lower panels).

Then we rationalized that as both CX3CL1 and IL15 were downregulated in recurrent PCa patients, more relevant measures of metastatic progression would include the traits of motility, adhesion, and adherence independent survival. For the first measure, we performed both transwell and scratch assays to determine motility of cells following incubation with CX3CL1 and IL15 containing stromal media. Both methods provided similar results, where CX3CL1 and IL15 significantly reduced the motility of LNCaP cells compared to control fibroblast conditioned media (**Figure 3,** transwell assay results not shown). Interestingly, the same factors had little effect on the motility of the BPH1 cells (**Figure 3**).

To determine the possibility of the role of the cytokines in regulating distant metastatic progression, we examined the ability of the cytokines to alter adhesion and survival in an adhesion free environment. The adhesion of LNCaP cells was significantly decreased by CX3CL1 and Il15 (**Figure 4**). However, aniokis sensitivity of LNCaP cells was significantly elevated by the same cytokines. We tested cell survival following incubation of the cells on non-adherent poly-HEMA coated plates – measured in two ways: by the apoptotic marker Anexin V and cell permeability by 7AAD staining, all quantitated by FACS analysis. We found that, BPH1 aniokis was not appreciably altered by the cytokines downregulated in PCa subjects developing recurrent progression. We found that CX3CL1 caused a significant increase in the early an early marker of apoptosis (extracellular surface expression of Annexin V) of LNCaP cells, compared to control (**Figure 5**). However, conditioned media containing both CX3CL1 and IL15 mediated cell death of LNCaP following growth in suspension (**Figure 6**). As there was elevated cell death

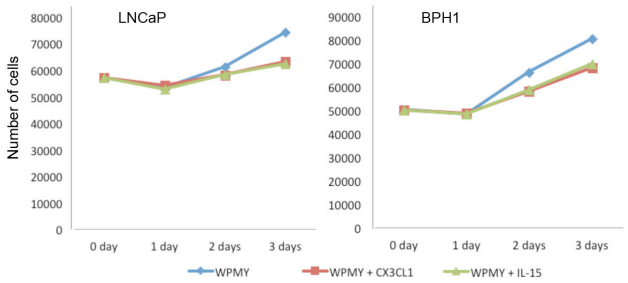

Figure 2. Addition of stromal conditioned media expressing CX3CL1 and IL15 reduces the proliferation of LNCaP and BPH1 cells. Recombinant cytokines expressed in 72hr conditioned media was added to prostatic cancer (LNCaP) and non-tumorigenic (BPH1) cells. Proliferation was measured by sequential counting over a 96 h period. The blue line indicatesconditioned media from GFP expressing control prostatic fibroblasts.

in IL15 treated cells over CX3CL1, it can be interpreted that both the cytokines down regulated in recurrent PCa subjects mediate sensitivity to aniokis. These results are in complete adherence to what would be expected if cells were to survive the duration in the circulation to a distant metastatic site.

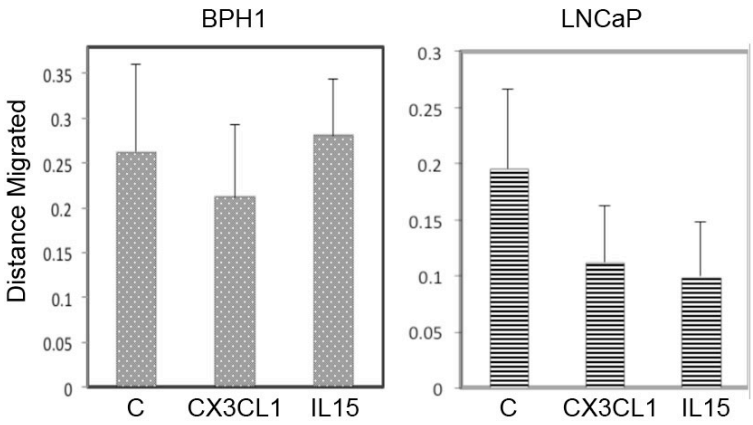

Figure 3. LNCaP cell motility is significantly diminished by CX3CL1 and IL15, based on scratch assay results. The motility of BPH1 cells was not affected similarly by the cytokines. (Similar results with transwell assays is not shown.)

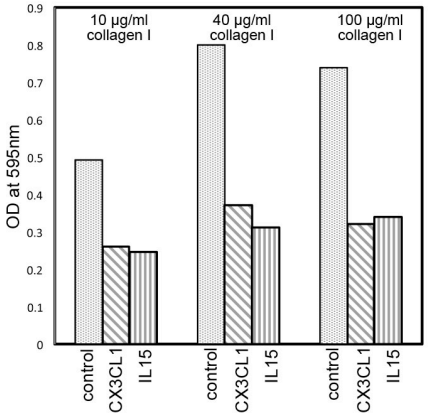

Figure 4. The adhesion of LNCaP cells to collagen I was diminished by treatment of stromal media containing CX3CL1 and IL15, compared to control.

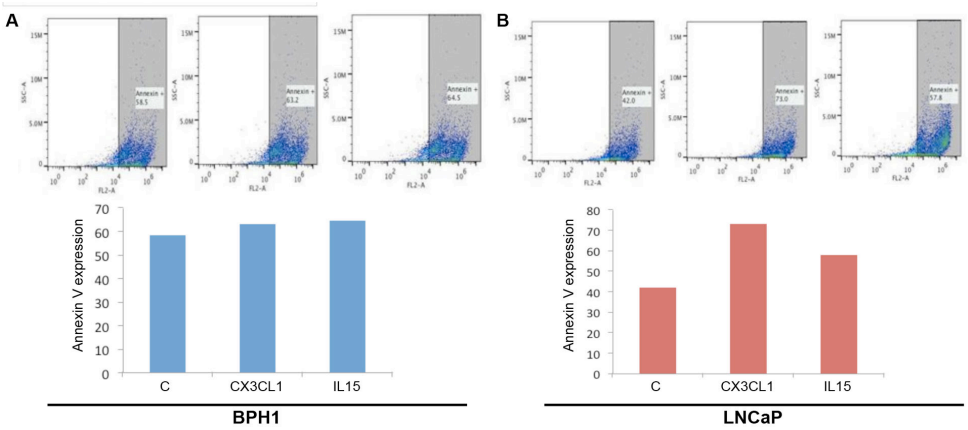

Figure 5. Annexin V FACS detection indicated CX3CL1 and IL15 caused aniokis sensitivity of LNCaP cells. There was little change in aniokis sensitivity of BPH1 cells by the same cytokines (A). CX3CL1 caused significant elevation AnnexinV detection in LNCaP cells grown in suspension, compared to control (B).

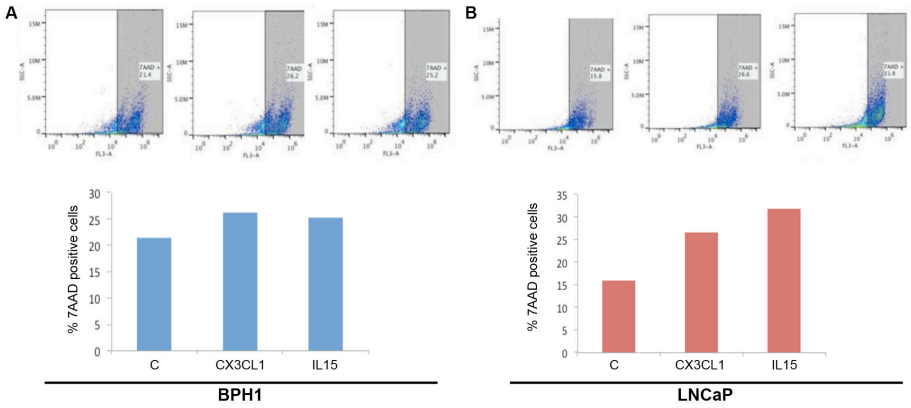

Figure 6. 7AAD FACS detection indicated CX3CL1 and IL15 caused aniokis sensitivity of LNCaP cells. There was little change in aniokis sensitivity of BPH1 cells by the same cytokines (A). Both cytokines caused elevated 7AAD detection in LNCaP cells grown in suspension (B).

c. **KEY RESEARCH ACCOMPLISHMENTS**

- We demonstrated that both CX3CL1 and IL15 paracrine signaling can reduce both the proliferation of both tumorigenic and non-tumorigenic prostatic epithelia.
- We demonstrated that both CX3CL1 and IL15 paracrine signaling has little effect on the motility of non-tumorigeneic BPH1 cells, but interestingly reduced tumorigenic LNCaP cell motility.
- We identified that both CX3CL1 and IL15 expression caused increased LNCaP sensitivity to aniokis, not BPH1 cells.

d. **REPORTABLE OUTCOMES**

Research

Publication

none.

Awards received based on work supported by this grant

Prostate Cancer Foundation 09/01/12-08/31/14

Isaacs/Karp/Bhowmick (PI) $1,000,000 direct cost

First-in-Man Clinical Studies of Mesenchymal Stem Cell Based Therapy for Prostate Cancer

The proposal tests the hypothesis that allogeneic human bone marrow-derived MSCs can be loaded with microparticles containing a drug so that when infused, they selectively deliver drug to metastatic sites of prostate cancer, thus sparing host toxicity. It involves the testing of the methodology in mouse models and performing phase zero studies in men with prostate cancer.

Products

CDNA construct, cell lines, and animal models developed

- Generated CX3CL1 over expressing human prostatic fibroblastic cells
- Generated IL15 over expressing human prostatic fibroblastic cells

e. **CONCLUSION**

The identification of CX3CL1, IL-15, and CCL4 as differentially expressed chemokines used to predict biochemical recurrence following prostatectomy supported the proposed studies where by CX3CL1 and IL-5 expression was associated with recurrent-free survival, where as CCL4 expression was associated with recurrence [12]. Thus the direct effects of CCL4 of cancer epithelia of varying metastatic potential were presented previously. In this report we focused on the biologic role of CX3CL1 and IL15 on prostate epithelia. Overwhelming data in this series of experiments suggest that these cytokines are not only downregulated in recurrent PCa subjects, but may in fact be anti-metastatic factors expressed by the stromal microenvironment. Previous studies have suggested the pro-tumorigenic properties of the tumor associated stromal fibroblastic cells. However, our data presented here support that the stroma can have an inhibitory role in metastatic progression. The elevated expression of extracellular matrix by carcinoma associated fibroblasts has been associated with restricting metastatic PCa progression [15]. However, here we show that specific cytokines can also have a restrictive role in metastatic progression. Importantly, CX3CL1 and IL15 had little effect of tumor cell proliferation, but rather the characteristics needed for distant metastatic progression. The on going xenografting experiments will tell if these results hold true in vivo.

The Prostate Cancer Foundation grant awarded due to the results generated due to the support of in this DOD application will enable “first in man” neo-adjuvant studies in PCa patients with locally advanced disease.

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